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Mouse C-Reactive Protein and Endotoxin-Induced Resistance

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ABSTRACT

PATTERSON, L. T. (University of Texas, Galveston), AND R. D. HIGGINBOTHAM. Mouse C-reactive protein and endotoxin-induced resistance. J. Bacteriol. 90:1520–1524. 1965.—The relationship between the level of C-reactive protein (CRP) in the sera of mice and resistance to *Staphylococcus aureus* infection after the injection of *Escherichia coli* endotoxin was studied. The CRP level was essentially unchanged at 6 hr after endotoxin, and resistance was slightly decreased. At 24 hr after endotoxin, both CRP levels and resistance were increased. Since the increase in the CRP level and resistance appeared to be associated, it was of interest that, when mouse CRP was tested for in vitro reactions with several strains of bacteria, cells of all species of gram-positive bacteria tested (including *S. aureus*) were agglutinated by CRP. *E. coli* was not agglutinated under the conditions of the test. It is proposed that mouse CRP is an opsonin, and possibly a lysin, and is involved in nonspecific resistance to infection with *S. aureus*.

Results of a recent study on staphylococcal arthritis in chickens suggested that the early production of C-reactive protein (CRP) is associated with resistance to *Staphylococcus aureus* (Patterson and Mora, 1965). The production of CRP in humans and Cx-reactive protein (CxRP) in rabbits can be induced by a variety of stimuli, including endotoxins (Hedlund, 1961). It is of interest, therefore, that many of these agents also can enhance resistance of mice to infection with *S. aureus* (Shilo and Wolman, 1958; Springer et al., 1961; Higginbotham, 1964) as well as with gram-negative bacteria (Rowley, 1964).

Considerable attention has been given to the possible role of both cellular and humoral factors in the endotoxin-induced nonspecific resistance in mice (Rowley, 1964; Shilo, 1959). However, there appear to have been no studies relating CRP to resistance in this species. The present study was conducted to determine whether the endotoxin-induced increase in resistance of mice to *S. aureus* infection was associated with the appearance of CRP-like substance in the sera of these animals. Evidence is presented that a substance that reacts with Cx-polysaccharide and antisera to human CRP was elevated in sera of mice during the early stage of increased resistance to infection. It was of interest that this protein caused the agglutination of cells of *S. aureus* as well as several other species of gram-positive bacteria.

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MATERIALS AND METHODS

Animals. Male mice of the Fairfield-Webster strain were used throughout this study. The animals were obtained from Euer's Farm, Austin, Tex., and were rested for at least 1 week prior to use. Food and water were available at all times. Animal weights ranged from 18 to 26 g. In individual experiments, weight differences varied from 2 to 4 g.

Treatment and challenge agents. Escherichia coli O55:B5 endotoxin and *Staphylococcus* A extract were obtained from Difco. For injection, the agents were suspended in isotonic, pyrogen-free saline (Baxter Laboratories, Morton Grove, Ill.) and administered by the intravenous route in a volume of 0.25 ml containing 100 μg of the appropriate bacterial extract. The relative effectiveness of these substances has been previously described (Higginbotham and Bass, 1964).

The challenge agent was the Fritchie strain of *S. aureus* prepared for use as previously reported (Higginbotham and Dougherty, 1955). After washing, the organisms were suspended in isotonic saline to a standard turbidity which represented approximately 10^9 viable cells per milliliter as determined by plate counts.

Bacterial agglutination by CRP. For testing of agglutinin activity, bacteria were prepared as follows: Fritchie and J. H. strains of *S. aureus*, *Bacillus subtilis*, and *E. coli* were grown overnight on nutrient agar slants, transferred to Brain Heart Infusion (BHI) broth (Difco) for 4 to 6 hr, centrifuged, washed in saline, and suspended in saline for testing. Tenfold dilutions were made and plated for viable counts. *Diplococcus pneumoniae*, Type 1 and rough, and *Streptococcus* spp. (β-hemolytic *S. pyogenes* and α-hemolytic species)

were grown overnight on BHI-blood agar, transferred to BHI broth for 4 to 6 hr, and prepared as above.

The CRP preparation used to test for bacterial agglutination was isolated from ascitic fluid produced by S_{37} tumor-bearing mice. The original fluid gave a positive reaction with Cx-polysaccharide and the latex agglutination test. The latter test was used to check subsequent steps in the purification of this material. The procedures for isolation of mouse CRP will be described in a later report. The CRP solution used for agglutination tests contained approximately 0.3 mg of protein per ml as determined by light adsorption at 280 mμ with bovine serum albumin as the standard.

To determine the effect of treatment on resistance, four groups of mice consisting of five animals each were injected intraperitoneally with 0.25 ml of saline containing one of a series of 0.5 $\log_{10}$ dilutions of the bacterial suspension. Data were recorded on the basis of numbers of animals surviving 24 hr after challenge. Previous studies have indicated that mortality is essentially complete at 24 hr (Higginbotham and Dougherty, 1955). Additional animals (five per treatment) were bled from the orbital sinus. The blood was allowed to clot, and the sera were analyzed separately for CRP by the latex agglutination method. The test was performed with the "CR test reagent" (Hyland Laboratories, Los Angeles, Calif.) on fourfold dilutions of the sera. The titer was reported as an index representing the reciprocal of the average end point dilutions.

Cx-polysaccharide of *D. pneumoniae* was prepared from a rough strain by the method described by Anderson and McCarty (1951) and was used for comparison with the latex reagent for the detection of CRP-like substances.

TABLE 1. *Effect of Escherichia coli endotoxin on CRP levels and on resistance to the lethal effects of Staphylococcus aureus infection*

Expt no.	Time of endotoxin injection prior to infection	CRP	LD_{50} ($\times 10^6$)
	hr		
1	0	2	3.8
	6	6	2.0
	24	40	12.2
2	0	4	6.1
	6	1	2.5
	24	52	25.0
3	0	2	1.8
	6	4	1.1
	24	26	5.3
4	0	2	6.5
	6	4	9.5
	24	54	36.0
Avg	0	2.5	4.5
	6	3.8	3.8
	24	43.0	19.6

TABLE 2. *Effect of Staphylococcus aureus endotoxin on CRP levels and on resistance to the lethal effects of S. aureus infection*

Time of injection	CRP	$LD_{50} \times 10^6$
hr		
Control	3.2	3
6	3.2	6
24	11.2	7

RESULTS

Cx-polysaccharide and latex antiserum reactions with mouse CRP. Results of previous work indicated that antisera to human CRP would react with chicken CRP (Patterson and Mora, 1964). Therefore, sera from mice previously treated with endotoxin were tested with several antisera against human CRP. It was observed that such sera reacted with the latex antisera and Cx-polysaccharide, whereas sera from "normal" mice did not react with either reagent, or reacted only at low dilutions.

To evaluate the comparative sensitivities of the latex antiserum and Cx-polysaccharide with test sera, blood was collected from 8 mice treated 24 hr previously with 100 μg of *E. coli* endotoxin. All sera gave positive reactions with latex antisera at the 1:4 dilution, but there was a lack of uniformity at the 1:16 dilution. When samples of the latter dilution of each sera were tested with the Cx-polysaccharide, the reactions were similar but slightly weaker than with the latex antiserum. Since this protein reacts with Cx-polysaccharide and has antigenic similarities to human CRP, it has the essential characteristics of CRP.

Effect of endotoxin on CRP and resistance to S. aureus. To examine the CRP response more closely, serum was obtained from groups of animals that had been injected with endotoxin 6 or 24 hr previously. The CRP titer of each sample was determined, and the average of each group was compared to that of the saline-treated mice. Each group consisted of five animals. Additional animals of each treatment group were included in the experiment to determine the effect of treatment on resistance to infection with *S. aureus*. The results of several experiments (Table 1) indicate that the effect of endotoxin on CRP levels was minimal at 6 hr after treatment, but was readily measurable by 24 hr; the average CRP titer increased about 20-fold. Resistance, as measured by the median lethal dose, usually was slightly decreased 6 hr after treatment and con-

TABLE 3. *Agglutination reactions of mouse CRP with certain bacteria*

Bacterial strain	Agglutination titer*	Bacteria per ml†
Staphylococcus aureus		
Fritchie	1:80	3.1×10^8
J.H	1:320	4.6×10^8
Diplococcus pneumoniae		
Smooth	1:20	2.5×10^6
Rough	1:1,280	5.6×10^6
Streptococcus		
α-Hemolytic	1:1,280	1.1×10^7
β-Hemolytic	1:1,280	3.2×10^8
Bacillus subtilis	1:320	3.0×10^6
Escherichia coli	No reaction	10.0×10^8

* Original CRP solution contained approximately 0.3 mg of protein per ml.

† Suspended at 10 to 30% T at 600 mμ.

sistently increased approximately three- to four-fold over that of the control group by 24 hr.

Similar, but less dramatic, results were obtained in a single experiment employing an extract of *S. aureus* as the stimulus (Table 2). The results indicate that extracts from either gram-positive or gram-negative bacteria can induce an increase in both CRP levels and resistance to *S. aureus* in mice.

Agglutination of bacteria by CRP. In view of the association between increased CRP and resistance to infection in treated mice, it was decided to test CRP for a direct effect on bacteria. In an associated study it was observed that ascitic fluid from tumor-bearing mice contained a high concentration of this substance. As relatively large volumes of this fluid could be readily obtained, it was used as the source material for isolation of CRP.

CRP material as detected by the latex test and Cx-polysaccharide was diluted in saline and mixed with the bacterial suspensions in final CRP dilutions of 1:5, 1:20, 1:80, 1:320, 1:1,280. The mixtures were shaken, placed in a water bath for 30 min, checked for agglutination, and placed in the refrigerator. They were checked for agglutination at intervals extending to 48 hr. Results of a representative experiment are presented in Table 3. The agglutination titers ranged from 1:20 with the smooth pneumococcus to 1:1,280 (the highest dilution tested) with rough *D. pneumoniae* and the streptococci. It is of interest that the specificity of the reaction, as determined by the time or titer at which agglutination occurred, did not appear to be of a higher degree for *D. pneumoniae* than for the other gram-positive bacteria tested.

DISCUSSION

This report confirms and extends the work of others on the increased resistance to *S. aureus* after endotoxin treatment (Shilo and Wolman, 1958; Springer et al., 1961; Higginbotham, 1964). In addition, it was observed that mouse CRP was increased during the 24-hr period when resistance to infection with *S. aureus* was also increased. This observation was consistent with previously reported associations between chicken CRP and resistance to intra-articular staphylococcal infection (Patterson and Mora, 1965) and CxRP and resistance to intradermal *S. aureus* infection in rabbits (Patterson, Salerno, and Higginbotham, 1964). The fact that a similar association between CRP and resistance to infection has been noted in three animal species indicates that it is not an unusual characteristic of a particular species. The agglutination of bacteria by mouse CRP suggests that CRP may have a direct role in resistance to infection.

Although the specific nature of the factors involved in increased resistance to *S. aureus* is still speculative (Shilo, 1959), endotoxin-enhanced resistance to infection with gram-negative bacteria has been ascribed, in part, to specific antibody (Whitby et al., 1961). However, the effects of specific immunization on a systemic challenge with *S. aureus* have ranged from a transient protection (Ekstedt, 1963) to decreased resistance due to hypersensitivity (Salerno and Higginbotham, 1965). It appears that the role of specific antibody in endotoxin-enhanced resistance to *S. aureus* is highly questionable.

It would appear that no single factor is responsible for endotoxin-increased resistance. Factors that may be involved include opsonins (Shilo, 1959), phagocytosis by leukocytes (Mulholland and Cluff, 1964) and reticuloendothelial (RES) cells (Biozzi, Benacerraf, and Halpern, 1955), and enzymatic activities enabling digestion to occur (Rutenberg et al., 1962) as well as an animal that is adequately supported by hormones, particularly the adrenal corticosteroids (Higginbotham, 1964).

Although "activation of the RES" is commonly invoked to explain endotoxin-enhanced resistance to infection, studies by Freedman and Sultzer (1964) indicate that clearance of carbon particles from the blood may be either increased or decreased, dependent upon the specific endotoxin preparation used, and that resistance to infection was increased regardless of the clearance effect.

However, activation of the RES may not be restricted to clearance effect alone but may also include stimulation of intracellular enzymatic activity. In this regard Suter and Ramseier (1964) described endotoxin-induced enzymatic changes in macrophages which may be conducive to enhanced resistance. Of related interest are the reports by Oroszlan, Mora, and Shear (1963) that a basic protein isolated from liver inactivated endotoxin and by Rutenberg et al. (1962) that endotoxin treatment caused an increase in the enzymatic degradation of endotoxin by spleen. Humoral substances other than specific antibody which might contribute to resistance have also been suggested. Skarnes and Chedid (1964) reported that serum from endotoxin-treated mice degraded endotoxin. Shilo (1959) reviewed the literature on several additional humoral activities. However, the humoral changes noted may reflect changes in function of RES or associated cells.

The primary effect of endotoxin is cell injury, and it is probable that the noted changes in cellular and humoral activities are essentially reactions to injury. That these reactions are not specific as to the inciting agent is apparent from the many irritants used to produce similar effects. As an example, Raskova (1964) reported that several of the effects of endotoxin were reproduced by phenol and other relatively simple compounds. The only apparent common property of the agents tested and endotoxin was tissue irritation.

The CRP response is a part of the response to injury. The serum CRP level is apparently related to the degree of injury and not to the nature of the injurious agent (Hedlund, 1961). The search for a function for CRP has attracted the attention of investigators for several years (Anderson and McCarty, 1951). Wood (1951) reported that CRP increased the rate of migration of leukocytes from the buffy coat. In a later paper he reported that the CxRP level after antigen injection was positively correlated with subsequent antibody production (Wood, 1953). However, Good, Bridges, and Condie (1960) reported that agammaglobulinemic patients produced CRP normally. Conversely, Hokama, Coleman, and Riley (1960) reported that the CxRP response could be suppressed in rabbits without inhibition of antibody production. It would thus appear that the two responses are not interdependent.

In view of the agglutination of bacteria by CRP in the present study, it is of interest that Jacox (1950) studied the obvious association between the CRP level and bactericidal activity of serum during acute infections. In his studies, the levels of these activities were not always parallel, and attempts to adsorb CRP did not remove the bactericidal activity. On this basis he concluded that CRP and the bactericidal factor were different. However, current studies in this laboratory (Patterson, Harper, and Higginbotham, 1965) suggest that isolated CRP possesses lytic activity for *S. aureus*.

In the present study, an association between serum CRP levels and resistance to infection with *S. aureus* was noted. When isolated mouse CRP was tested for a direct effect on the cells of several strains of bacteria, agglutination was found to occur with all gram-positive bacteria tested. This included two strains of *S. aureus* of known pathogenicity. The agglutination reaction indicates that changes occurred at the cell surface, and it is possible that the agglutination is caused by adsorption of CRP to cells. It is also possible that CRP is enzymatic in nature and can degrade certain components of the bacterial cell, thereby changing the stability of the suspension. Presumably, either of these effects could render the cells more easily phagocytized and, possibly, digested. In this regard, Hokama, Coleman, and Riley (1962) reported that pretreatment of bacteria with human CRP increased phagocytosis. It would thus appear that CRP may function as an opsonin or lysin and contribute to the endotoxin-enhanced, nonspecific resistance to infection with *S. aureus*.

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LITERATURE CITED

ANDERSON, H. C., AND M. MCCARTY. 1951. The occurrence in the rabbit of an acute phase protein analogous to human C-reactive protein. J. Exptl. Med. **93**:25–36.

BIOZZI, G., B. BENACERRAF, AND B. N. HALPERN. 1955. The effect of *Salm. typhi* and its endotoxin on the phagocytic activity of the reticuloendothelial system in mice. Brit. J. Exptl. Pathol. **36**:226–235.

EKSTEDT, R. D. 1963. Studies on immunity to staphylococcal infection in mice. I. Effect of dosage, viability, and interval between immunization and challenge on resistance to infection following injection of whole cell vaccines. J. Infect. Diseases. **112**:143–151.

FREEDMAN, H. H., AND B. M. SULTZER. 1964. Aspects of endotoxin tolerance: phagocytosis and specificity, p. 537–545. *In* M. Landy and W. Braun [ed.], Bacterial endotoxins. Rutgers Univ. Press, New Brunswick, N.J.

GOOD, R. A., R. A. BRIDGES, AND R. M. CONDIE. 1960. Host-parasite relationships in patients with dysproteinemias. Bacteriol. Rev. **24**:115–131.

HEDLUND, P. 1961. Clinical and experimental

studies on C-reactive protein (acute phase protein). Acta Med. Scand. Suppl. **361**:1–71.

HIGGINBOTHAM, R. D. 1964. Endotoxin enhanced resistance in adrenalectomized mice. Federation Proc. **23**:564.

HIGGINBOTHAM, R. D., AND J. A. BASS. 1964. Endotoxic properties of an extract of *S. aureus*. Proc. Soc. Exptl. Biol. Med. **116**:26–30.

HIGGINBOTHAM, R. D., AND T. F. DOUGHERTY. 1955. Mechanism of protective effect of hydrocortisone in staphylococci infected adrenalectomized mice. Proc. Soc. Exptl. Biol. Med. **90**:253–258.

HOKAMA, Y., M. K. COLEMAN, AND R. F. RILEY. 1960. Effects of drugs on Cx-protein responses in the rabbit. Proc. Soc. Exptl. Biol. Med. **105**:510–514.

HOKAMA, Y., M. K. COLEMAN, AND R. F. RILEY. 1962. *In vitro* effects of C-reactive protein on phagocytosis. J. Bacteriol. **83**:1017–1024.

JACOX, R. F. 1950. The activating effect of calcium on a bactericidal substance for *B. subtilis*. J. Exptl. Med. **92**:101–111.

MULHOLLAND, J. H., AND L. E. CLUFF. 1964. The effect of endotoxin upon susceptibility to infection: the role of the granulocyte, p. 211–229. *In* M. Landy and W. Braun [ed.], Bacterial endotoxins. Rutgers Univ. Press, New Brunswick, N.J.

OROSZLAN, S. I., P. T. MORA, AND M. J. SHEAR. 1963. Reversible inactivation of an endotoxin by intracellular protein. Biochem. Pharmacol. **12**:1131–1146.

PATTERSON, L. T., AND E. C. MORA. 1964. Occurrence of a substance analogous to C-reactive protein in the blood of the domestic fowl. Texas Rept. Biol. Med. **22**:716–721.

PATTERSON, L. T., S. J. SALERNO, AND R. D. HIGGINBOTHAM. 1964. The relationship of C-reactive protein-like substances to endotoxin-enhanced resistance. Texas Rept. Biol. Med. **22**:221.

PATTERSON, L. T., J. M. HARPER, AND R. D. HIGGINBOTHAM. 1965. Mouse C-reactive protein and nonspecific resistance to infections. Federation Proc. **24**:699.

PATTERSON, L. T., AND E. C. MORA. 1965. The C-reactive protein response and disease resistance in the domestic fowl. Texas Rept. Biol. Med. **23**:600–606.

RASKOVA, H. 1964. Nonspecific endotoxin-like resistance induced by simple chemical compounds, p. 546–561. *In* M. Landy and W. Braun [ed.], Bacterial endotoxins. Rutgers Univ. Press, New Brunswick, N. J.

ROWLEY, D. 1964. Endotoxin-induced changes in susceptibility to infections, p. 359–372. *In* M. Landy and W. Braun [ed.], Bacterial endotoxins. Rutgers Univ. Press, New Brunswick, N.J.

RUTENBERG, S. H., E. E. SMITH, A. M. RUTENBERG, AND J. FINE. 1962. Degradation of endotoxin by splenic extracts. Antimicrobial Agents and Chemotherapy—1961, p. 142–147.

SALERNO S., AND R. D. HIGGINBOTHAM. 1965. Effect of vaccination on resistance of mice to local and systemic challenge with *Staphylococcus aureus*. Bacteriol. Proc., p. 62.

SHILO, M. 1959. Nonspecific resistance to infection. Ann. Rev. Microbiol. **13**:255–278.

SHILO, M., AND B. WOLMAN. 1958. Activities of bacterial levans and of lipopolysaccharides in the processes of inflammation and infection. Brit. J. Exptl. Pathol. **39**:652–660.

SKARNES, R. C., AND L. C. CHEDID. 1964. Biological degradation and inactivation of endotoxin (chromate-labelled), p. 575–587. *In* M. Landy and W. Braun [ed.], Bacterial endotoxins. Rutgers Univ. Press, New Brunswick, N.J.

SPRINGER, G. F., E. STEERS, S. DHANAMITTA, J. STINNETT, AND P. GYORGY. 1961. Protection of mice against lethal *Staphylococcus* infection by *Escherichia coli* O_{86} fractions. Science **134**:335–336.

SUTER, E., AND H. RAMSEIER. 1964. Cellular reactions in infection. Advan. Immunol. **4**:117–173.

WHITBY, J. L., J. G. MICHAEL, M. W. WOODS, AND M. LANDY. 1961. Possible mechanisms whereby endotoxins evoke increased nonspecific resistance to infection. Bacteriol. Rev. **25**:437–446.

WOOD, H. F. 1951. Effect of C-reactive protein on normal human leucocytes. Proc. Soc. Exptl. Biol. Med. **76**:843–847.

WOOD, H. F. 1953. The relationship between the acute phase response and antibody production in the rabbit. J. Exptl. Med. **98**:311–319.